Conditionally immortalized human glomerular endothelial cells expressing fenestrations in response to VEGF

SC Satchell[1], CH Tasman[1], A Singh[1], L Ni[1], J Geelen[2], CJ von Ruhland[3], MJ O'Hare[4], MA Saleem[1], LP van den Heuvel[2] and PW Mathieson[1]

[1]Academic Renal Unit, University of Bristol, Southmead Hospital, Bristol, UK; [2]Department of Paediatrics, Radboud University Nijmegen Medical Centre, HB Nijmegen, The Netherlands; [3]Medical Microscopy Sciences, School of Medicine, Cardiff University, Heath Park, Cardiff, UK and [4]Ludwig Institute for Cancer Research-University College London Breast Cancer Laboratory, Department of Surgery, Royal Free and University College School of Medicine, London, UK

Glomerular endothelial cells (GEnC) are specialized cells with important roles in physiological filtration and glomerular disease. Despite their unique features, GEnC have been little studied because of difficulty in maintaining them in cell culture. We have addressed this problem by generation of conditionally immortalized (ci) human GEnC using technology with which we have previously produced ci podocytes. Primary culture GEnC were transduced with temperature-sensitive simian virus 40 large tumour antigen and telomerase using retroviral vectors. Cells were selected, cloned, and then characterized by light and electron microscopy (EM), response to vascular endothelial growth factor (VEGF), and tumour necrosis factor (TNF)α, expression of endothelial markers by focused gene array, immunofluorescence and Western blotting, and formation and behaviour of monolayers. CiGEnC proliferated at the permissive temperature (33°C) and became growth arrested at the non-permissive temperature (37°C). CiGEnC retained morphological features of early-passage primary culture GEnC up to at least p41, confirming successful immortalization. EM demonstrated fenestrations, increased in number by VEGF. mRNA analysis confirmed expression of the endothelial markers platelet endothelial cell adhesion molecule 1, intercellular adhesion molecule 2, VEGF receptor 2, and von Willebrand factor, validated by immunofluorescence and Western blotting. CiGEnC also expressed Tie2, and TNFα upregulated E-selectin. CiGEnC formed monolayers with barrier properties responsive to cyclic adenosine 3′,5′ monophosphate (cAMP) and thrombin. CiGEnC retain the markers and behaviour of primary culture GEnC. They express fenestrations which are upregulated in response to VEGF. These cells are a unique resource for further study of GEnC and their roles in glomerular filtration, glomerular disease, and response to glomerular injury.

Kidney International (2006) **69,** 1633–1640. doi:10.1038/sj.ki.5000277; published online 22 March 2006

KEYWORDS: glomerular endothelial cell; vascular endothelial growth factor; fenestration

The glomerular microcirculation, unlike other capillary circulations, is highly permeable to water and small solutes, yet, like other capillaries, is relatively impermeable to macromolecules. These properties are essential for filtration function and are a result of the unique three-layer structure of the glomerular capillary wall: the endothelium with its glycocalyx, the glomerular basement membrane, and podocytes. Much interest has recently focused on the podocyte and particularly the slit diaphragm as a possible site of a final barrier to macromolecular passage.[1] However, models indicate that all three layers have an important contribution and that their combined properties are more than simply the sum of the parts.[2,3]

Glomerular endothelial cells (GEnC) are highly specialized cells, which form a continuous inner layer of glomerular capillaries. Away from the nucleus the cytoplasm is attenuated to 200 nm and is punctuated by numerous fenestrae. These are circular transcellular pores, 60–80 nm in diameter,[4] which cover 20% of the endothelial surface[3] and are essential for high hydraulic conductivity. Previously, the fenestrations were thought of as empty and therefore providing little barrier to the passage of proteins.[5] However, new fixation techniques have allowed the demonstration of a glomerular endothelial glycocalyx of 200–400 nm in thickness, which covers fenestrae and interfenestral domains equally.[4] Although the exact nature of this GEnC glycocalyx is yet to be defined, these observations suggest that the contribution of GEnC to the permeability barrier to proteins may have been underestimated.[6]

GEnC are also likely to be important in glomerular disease. There is clear evidence of GEnC damage in conditions such as

Correspondence: *SC Satchell, Academic Renal Unit, University of Bristol, Southmead Hospital, Bristol BS10 5NB, UK. E-mail: s.c.satchell@bristol.ac.uk*

Received 23 June 2005; revised 11 October 2005; accepted 28 October 2005; published online 22 March 2006

haemolytic uraemic syndrome[7] and pre-eclampsia.[8] Diseases such as haemolytic uraemic syndrome, vasculitis, and anti-glomerular basement membrane disease involve destruction of glomerular capillaries and experimental models indicate that recovery is dependent on GEnC angiogenesis.[9–11] Systemic conditions such as diabetes, hypertension, and sepsis are associated with microalbuminuria and, although the mechanism of this link is not known, the generalized endothelial dysfunction that accompanies these conditions suggests a contribution from a GEnC lesion.[12,13]

In addition to their unique structure and function, GEnC also have a particular embryological origin, arising by a combination of vasculogenesis from mesenchymal precursors (predominantly) and in-growth of existing vessels.[14,15] Furthermore increasing evidence indicates that EnC from different species, organs, and types of vessels have different properties and behaviours.[16–18]

All these considerations indicate the importance of the study of human GEnC to enable complete understanding of glomerular filtration, glomerular disease, response to glomerular injury, and the potential for therapeutic manipulations in these contexts. While certain responses are most appropriately examined *in vivo*, cell culture allows careful analysis of the behaviour of individual cell types and the actions and interactions of particular mediators. Historically, GEnC have been difficult to grow in culture and have therefore been little studied. We have now addressed this problem using conditional immortalization (ci) technology, such as we have successfully applied previously in cultured podocytes.[19] While a number of groups have now successfully cultured rodent or bovine GEnC,[20–23] there are few reports convincingly demonstrating isolation of human GEnC.[7] In this case GEnC were cultured from glomeruli sieved from normal renal cortex. The technique involved partial collagenase digestion of the glomerulus, coating of culture plates with extracellular matrix components, use of medium with EnC growth factors, and purification of GEnC from contaminating cells using an immunomagnetic bead selection technique. Human GEnC, similarly derived from glomeruli decapsulated by sieving, have also become available from a commercial source and we have previously characterized these cells in detail.[24] Other described culture methods for 'glomerular' EnC, which begin with lysing whole cortex,[25,26] result in a mixture of EnC, only some of which are glomerular.

When successfully isolated, primary culture GEnC form a monolayer of polygonal cells, which express typical EnC-specific markers including von Willebrand factor (vWF), platelet endothelial cell adhesion molecule 1 (PECAM1, CD31), and vascular endothelial (VE)-cadherin (CD144), and growth factor receptors Tie2 and vascular endothelial growth factor receptor 2 (VEGFR2).[7,24] These cells, like other EnC, respond to inflammatory mediators, including tumour necrosis factor (TNF)α, by upregulation of the adhesion molecule E-selectin (CD62E), and they form monolayers in culture whose permeability properties are responsive to cyclic adenosine 3′,5′ monophosphate (cAMP) and thrombin. Human primary culture GEnC express some fenestrations in culture,[7] while bovine GEnC do not express fenestrations in static culture but can be induced by shear stress.[27]

The usefulness of primary culture human GEnC for tissue culture studies is limited by early onset of senescence. Here we describe the ci of human GEnC. We have used a technique restoring functional telomerase activity and introducing the simian virus 40 large tumour antigen (SV40LT), since it has been shown that human EnC require both these elements for successful immortalization.[28] A temperature-sensitive (ts) SV40LT construct was used to allow ci, that is, enhanced proliferation at a permissive temperature, while the SV40LT element can be 'switched off' by transfer to a non-permissive temperature. At this temperature, cells take on a mature phenotype not seen in cells constitutively expressing SV40LT. This approach has been used successfully in alveolar bone marrow cells,[29] breast microvascular EnC and fibroblasts,[28] and in podocytes in our laboratory.[19]

RESULTS

Cell culture and phase-contrast microscopy

Clones of ciGEnC successfully transduced with both SV40LT and the essential catalytic subunit of human telomerase (hTERT) were antibiotic-resistant and proliferated at 33°C with growth arrest occurring on transfer to 37°C. CiGEnC at both 33°C and 37°C retained features of early-passage primary culture GEnC, including small size, homogeneity, and formation of 'cobblestone' monolayers up to at least passage 41 (Figure 1), while primary culture GEnC became senescent by passage 8 (not shown). CiGEnC at 37°C were used in experiments after at least 5 days at the non-permissive temperature.

Scanning electron microscopy

Scanning electron microscopy of ciGEnC monolayers demonstrated the presence of cytoplasmic pores of 50–400 nm

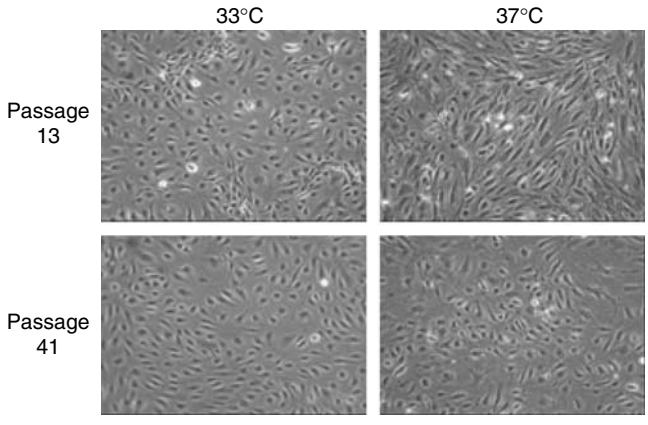

Figure 1 | Phase-contrast microscopy showing ciGEnC at early (13) and late (41) passage and at the permissive (33°C) and non-permissive (37°C) temperatures. CiGEnC maintain morphological appearances of primary culture GEnC (not shown) at late passage. Original magnification × 100.

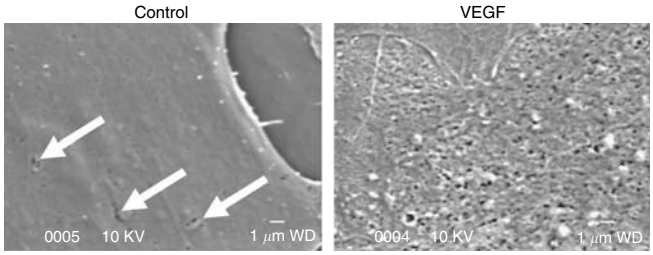

Figure 2 | SEM showing the presence of fenestrations 50–400 nm in diameter (arrows) in ciGEnC at 37°C under usual conditions (control) and the striking increase in their number after 24 h treatment with 100 ng/ml VEGF. Bar = 1 μm.

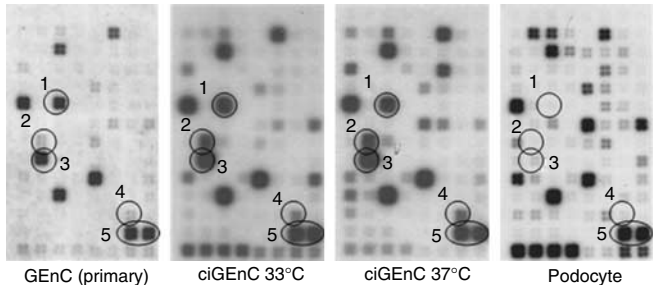

Figure 3 | Focused gene array images derived from mRNA extracted from primary culture GEnC, ciGEnC at 33°C, and after 7 days at 37°C and podocytes (control). Pattern of expressed mRNA detected is similar for all three GEnC types, but quite different for podocytes. Examples of groups of four quadruplicate dots representing endothelial cell-specific markers are indicated by numbered circles; 1: ICAM2, 2: VEGFR2, 3: PECAM1, 4: vWF. One of the control genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is also indicated.[5]

in diameter which were taken to represent fenestrations, although they were slightly larger than the 60–80 nm estimated from *ex vivo* studies.[3,4] These were greatly increased in number by VEGF (Figure 2).

Gene expression analysis by cDNA array

mRNA analysis demonstrated that the overall expression patterns of genes represented on the array used were very similar for primary and ciGEnC at both permissive and non-permissive temperatures, but quite different from the expression pattern for the podocytes used as a control (Figure 3). The analysis confirmed the expression of the EnC-specific markers PECAM1, intercellular adhesion molecule 2 (ICAM2, CD102), VEGFR2, and vWF in the three GEnC groups, but not in podocytes (Table 1). There were some differences between the three GEnC groups in levels of mRNA expression in these experiments, for example, relatively higher expression of VEGFR2 and vWF in ciGEnC. Interestingly, some genes involved in extracellular matrix production and regulation (plasminogen activator inhibitor-1, thrombospondin, fibronectin) were highly expressed in all GEnC and podocytes (Table 1).

Table 1 | Relative expression of endothelium-specific and matrix-related genes in GEnC, selected from genes analysed by focused gene array

	Gene expression level (ratio of gene:GAPDH dot density) for different cell types			
Gene	Primary GEnC	CiGEnC 33°C	CiGEnC 37°C	Podocytes
Endothelium-specific				
PECAM1	1.12	0.93	0.98	0.08
ICAM2	1.04	1.08	1.26	0.02
VEGFR2	0.27	0.75	0.81	0.01
vWF	0.17	0.48	0.66	0.05
Matrix-related				
PAI-1	1.15	1.08	1.15	1.04
Thrombospondin	1.05	1.11	1.13	1.08
Fibronectin	1.15	1.05	1.22	1.07

ciGENC, conditionally immortalized glomerular endothelial cells; ICAM, intercellular adhesion molecule; PAI, plasminogen activator inhibitor; PECAM, platelet endothelial cell adhesion molecule; VEGFR, vascular endothelial growth factor receptor; vWF, von Willebrand factor.

Immunofluorescence of ciGEnC monolayers and cell-based fluorescence immunoassay

CiGEnC expressed the EnC-specific markers PECAM1, vWF, and VE-cadherin comparable in distribution and level of expression to primary culture GEnC (Figure 4). Podocytes were, as expected, negative for these markers. Treatment of ciGEnC with TNFα caused a dose-dependent increase in expression of E-selectin by cell-based fluorescence immunoassay (Figure 5), but had no effect on binding of an irrelevant primary antibody (not shown). TNFα (10 ng/ml) caused a 3.2-fold increase in E-selectin expression over 6 h.

Western blotting

Western blotting confirmed that ciGEnC retain the expression of the important EnC-specific molecules PECAM1, ICAM2, VEGFR2, vWF, VE-cadherin, and Tie2 at both 33°C and 37°C (Figure 6). These results correspond to and confirm similar gene array results for PECAM1, ICAM2, VEGFR2, and vWF. There were comparable levels of expression in primary and ciGEnC, although as above there appeared to be greater expression of VEGFR2 and vWF in ciGEnC.

Western blotting also confirmed expression of the SV40LT antigen in ciGEnC at 33°C and its silencing by transfer of cells to 37°C. Time-course analysis showed SV40LT levels rapidly reducing within 24 h with a greater than 30-fold reduction 5 days after transfer than at 33°C (Figure 7). Cells were taken to be quiescent by day 5 and were used in experiments after that time point.

Trans-endothelial electrical resistance and effects of thrombin and cAMP

CiGEnC monolayers at 33°C reached a mean *trans*-endothelial electrical resistance (TEER) of 20–25 Ω at 6 days post-seeding, after which the TEER plateaued (data not shown). Monolayers transferred to 37°C on day 6 maintained a similar TEER for a further 7 days. Increasing the effective

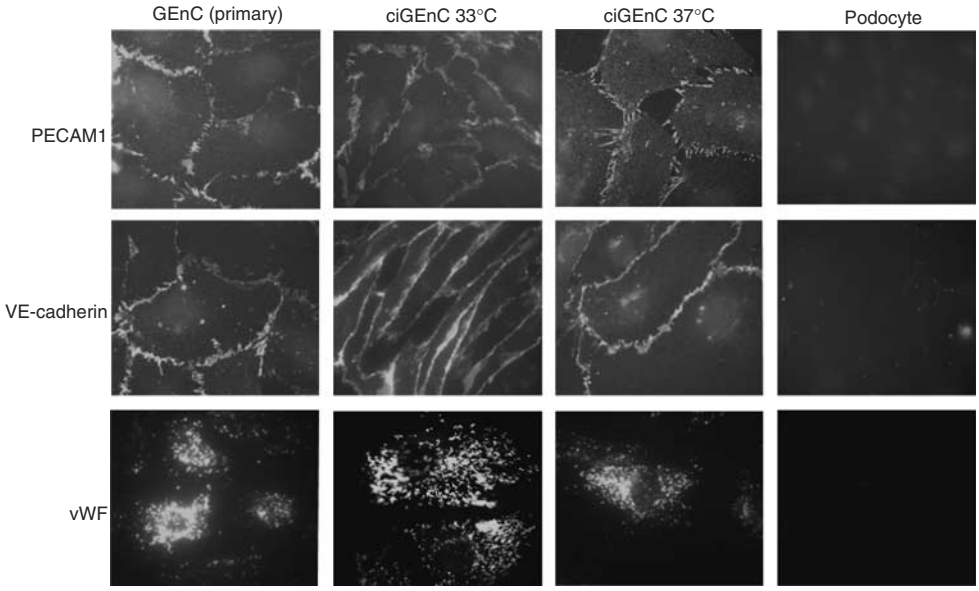

Figure 4 | Immunofluorescence microscopy showing expression of EnC-specific markers PECAM-1, VE-cadherin, and vWF by primary culture GEnC, ciGEnC at 33°C, and after 14 days at 37°C. Podocytes used as a control are negative for these EnC markers as expected. Original magnification ×1000.

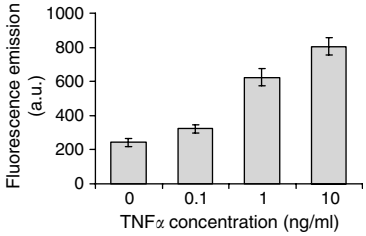

Figure 5 | Chart showing the effect of TNFα on e-selectin expression by ciGEnC in a cell-based FIA. CiGEnC at 37°C were incubated with TNFα at various concentrations or control medium for 6 h before fixing and labelling for E-selectin. E-selectin expression is proportional to fluorescence emission. Bars show mean ± s.e., $n = 15$, $P < 0.0001$ by analysis of variance.

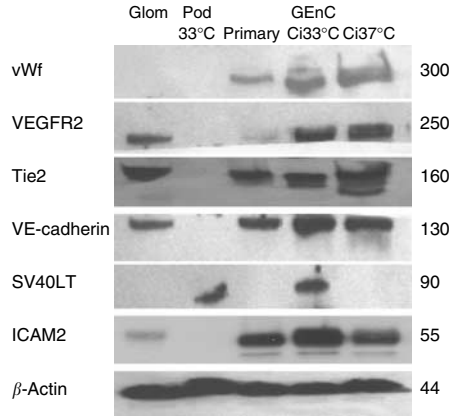

Figure 6 | Western blotting analyses of protein extracted from glomeruli sieved from normal renal cortex (Glom), ci podocytes (Pod) at 33°C, primary culture GEnC and ciGEnC at 33°C, and after 10 days at 37°C, demonstrating the expression of EnC-specific markers in all GEnC lanes and of SV40LT in ci podocytes and ciGEnC at 33°C. Images were derived from identical gels, with each lane loaded with the same amount (10 μg) of the same protein samples. Actin bands confirm the loading of comparable amounts of protein. Numbers indicate the expected molecular weight of bands and correspond to molecular weights of marker proteins (not shown).

intracellular concentration of cAMP by use of the 'cAMP medium' increased the mean TEER of ciGEnC monolayers at 37°C by 6.3 Ω, while thrombin decreased TEER by 7.6 Ω over 1 h relative to controls (Figure 8).

DISCUSSION

We have described for the first time the generation of immortalized human GEnC. Significantly, this immortalization is conditional by use of a temperature-sensitive transgene encoding SV40LT. This allows both an unlimited replication potential and return of cells to a non-proliferative state by transfer to the non-permissive temperature of 37°C. Western blotting confirmed rapid reduction of SV40LT levels after transfer to 37°C. This is consistent with previous observations on other cells, ci in a similar way, which show rapid SV40LT loss on transfer to the non-permissive temperature, leading to growth arrest, while metabolic activity is maintained.[30] Indeed the tsA58 mutant was selected as the source of SV40LT for this property of rapid degradation at the non-permissive temperature.[30] In addition, we have also incorporated a transgene encoding hTERT, shown to be essential for successful immortalization of human EnC in previous studies.[28]

We have directly compared ciGEnC with primary culture human GEnC, previously characterized in detail,[24] and shown that they retain similar morphological appearances at both non-permissive and permissive temperatures. Similar

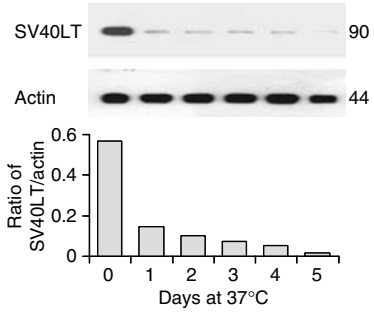

Figure 7 | Western blotting analyses (top panel) and ratio of band density of SV40LT to actin (lower panel) of protein extracted from ciGEnC at 33°C (day 0) and at 37°C (day 1–5), demonstrating the rapid loss of expression of SV40LT on transfer to the non-permissive temperature. Images were derived from identical gels with each lane loaded with the same amount (10 μg) of the same protein samples. Actin bands confirm the loading of comparable amounts of protein. Numbers indicate the expected molecular weight of bands and correspond to molecular weights of marker proteins (not shown).

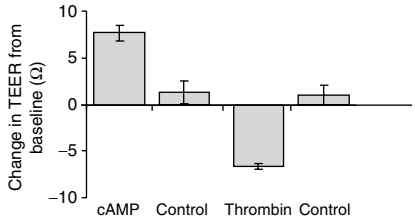

Figure 8 | Chart showing the results of separate experiments examining the effect of cAMP medium (containing 2 μM RO-20-1724 and 30 μM 8-(4-chlorophenylthio)-cAMP) or thrombin (1 U/ml) on TEER of ciGEnC monolayers after 1 h. Bars show mean ± s.e., $n = 3$, $P < 0.05$ for effect of each mediator, P-values by t-test. cAMP medium increased TEER by 6.3 Ω, while thrombin decreased TEER by 7.6 Ω relative to control.

patterns of mRNA expression were seen using a focused gene array including the expression of EnC-specific markers PECAM1, ICAM2, VEGFR2, and vWF. The expression of these markers was validated at the protein level by Western blotting and/or immunofluorescence. GEnC were also shown to express VE-cadherin and Tie2 at comparable levels in primary and ci cells by Western blotting. Further interesting data from the gene array experiments show that GEnC, like podocytes in culture, expressed high levels of mRNA for proteins which are extracellular matrix components (fibronectin, thrombospondin) or involved in regulation of biological reactions occurring in it (plasminogen activator inhibitor-1). This may be important in the production and remodelling of the glomerular basement membrane *in vivo*.

CiGEnC retained EnC behaviours which are important *in vivo*, and hence will be important in their further use for *in vitro* studies. Like primary culture GEnC, ciGEnC upregulated E-selectin in response to the inflammatory mediator TNFα and they formed restrictive monolayers in culture. These monolayers were responsive to both cAMP and thrombin as we have previously shown for primary culture GEnC, and comparable to EnC of other types.[24]

For the first time in cultured GEnC we have shown the upregulation of fenestrations in response to VEGF. The presence of fenestrations *in vivo* is essential for the high hydraulic conductivity of the glomerular filtration barrier and hence glomerular filtration. The fact that similar structures are expressed *in vitro* suggests that observations on the barrier-forming properties of these cells will be relevant to pathophysiology of the intact glomerulus. Expression of VEGF receptors and responsiveness to this mediator are particularly noteworthy in this respect as evidence suggests the importance of VEGF in normal glomerular physiology in maintenance of the endothelium as well as in glomerular disease.[31] It has long been suggested that VEGF, produced by podocytes, may induce GEnC fenestrations.[5,32] Although it is yet to be proven that this occurs *in vivo*, these *in vitro* observations lend further weight to this hypothesis and are consistent with our previous observations of VEGF reducing the TEER of primary culture GEnC monolayers.[24]

The ready availability of large numbers of human GEnC now opens the door for detailed *in vitro* studies. Formation of restrictive monolayers indicates that these cells will be useful for further investigation of GEnC barrier function, including analysis of the role of glycocalyx. This will enable greater understanding of the contribution of GEnC to physiological glomerular permselectivity and to disturbances in disease states. This includes intrinsic glomerular diseases where the exact mechanisms of proteinuria are yet to be elucidated, and also those systemic conditions in which there is a more modest increase in glomerular permeability resulting in microalbuminuria. Study of GEnC in culture may illuminate the important conundrum of why microalbuminuria is a powerful independent risk factor for disease in other vessels and hence for cardiovascular disease.[33] Increasing evidence indicates the importance of interactions between GEnC and podocytes in filtration barrier function in health and disease.[24] As we have previously derived ci podocytes, we can now study the two cell types separately and together in further *in vitro* analyses of factors involved in glomerular permeability.

In summary, we have successfully addressed the problem of poor replicative potential of human GEnC *in vitro* by development of ciGEnC, which represent a unique resource for further study. The importance of studying the relevant cell type in tissue culture studies is clear and hence these cells significantly advance our ability to understand the human renal glomerulus through *in vitro* investigations. The cells retain the characteristics of primary culture cells, including aspects of their highly specialized *in vivo* ultrastructure such as fenestration formation. These cells have been developed at a time of rapidly increasing understanding of the ultrastructural and molecular basis of glomerular permselectivity in health and disease through the detailed study of podocytes.[34] In the light of observations indicating that the glomerular endothelium also plays a vital role, a complementary study of these ciGEnC promises to further enhance this progress.

MATERIALS AND METHODS

Cell culture

Primary culture GEnC derived from isolated human glomeruli were prepared as described previously.[7] Additional primary culture GEnC derived from glomeruli isolated from normal human kidney and decapsulated by sieving (according to the supplier's data sheet) were obtained at passage 2 from the Applied Cell Biology Research Institute (ACBRI, Kirkland, WA, USA) as previously characterized in detail.[24] Cells were cultured in endothelial growth medium 2 – microvascular (EGM2-MV, Cambrex, Wokingham, UK) containing foetal calf serum (5%) and growth factors as supplied, excepting VEGF unless otherwise stated. Human ci podocytes were cultured in RPMI 1640-based medium as described previously.[19] A mouse fibroblast cell line (NIH 3T3, obtained from the European Collection of Cell Cultures via Sigma Chemical Co., St Louis, MO, USA) was cultured in the same RPMI-based medium.

Ci – retroviral construct, generation of amphotropic viruses, and virus infection

Two packaging cell clones producing helper-free amphotropic murine-leukemia-virus particles as described previously were used.[28] Briefly, the first, based on the packaging cell line PA317, produced viral particles containing an SV40LT antigen gene containing both tsA58 and the U19 mutations. The second, based on the packaging cell line TEFLY-A, produced virus particles containing hTERT. Cultures of primary human GEnC (at passage 4–6) were simultaneously infected with retrovirus-containing supernatants from the two packaging cell lines. Midconfluence proliferating cultures were exposed to freshly thawed filtered (0.45 μm) supernatant mixed 1:1 with growth medium plus 8 μg/ml polybrene for 18 h before being replaced with usual growth medium. The cells were allowed to reach confluence at 37°C. Cells transduced with the SV40LT construct were selected with 0.5 mg/ml G418 (Life Technologies BRL, Paisley, UK) and cells were placed at 33°C. Cells were maintained at this temperature for the remainder of the procedure. At 5 days the medium was changed for normal growth medium. After 2 weeks clones of healthy cells selected for the SV40LT construct began to appear. Some of these patches were removed from the flask using cloning rings and transferred to fresh flasks. After reaching confluence, cells also transduced with hTERT were selected with 25 μg/ml hygromycin (Sigma) for 5 days, after which surviving cells were allowed to approach confluence in standard medium.

Subcloning of cell lines

Flasks of cells were selected by phase-contrast microscopy for dilution cloning and further characterization on the basis of the greatest similarity with primary culture cells, homogeneity, and continued proliferation at 33°C. Fibroblasts, to be used as feeder cells, were treated with mitomycin-C (0.25 μg/ml, Sigma) for 12 h to render them non-proliferative. After washing in usual growth medium, and a further 48 h in culture, fibroblasts were plated at 10 000 cells/cm^2, resulting in a 70% confluent cell layer. After another 24–48 h, GEnC were seeded into the same plates at concentrations of 10 or 50 cells/ml. Single-cell clones were selected at 21–28 days using cloning rings and were transferred to fresh culture plates.

Propagation of cells

Cells were grown to confluence at 33°C, trypsinized, and reseeded in fresh flasks at a dilution between 1:3 and 1:5. Cells were grown to confluence before thermoswitching to 37°C. This was chosen as the non-permissive temperature, as in previous studies EnC containing the tsA58 SV40LT mutant were fully arrested at this temperature, although the full non-permissive temperature for tsA58 is 39.5°C.[28]

Phase-contrast microscopy

CiGEnC at various passages were seeded in flasks at subconfluent density, placed at either 33°C or 37°C, and morphology was examined by phase-contrast microscopy over time. Appearances were compared with primary culture cells and digital images were acquired.

Scanning electron microscopy

Cells grown to confluence on plastic (Thermanox, Nalge Nunc International, Rochester, NY, USA) coverslips were fixed in 2.5% glutaraldehyde in 0.1 M Sorenson's phosphate buffer, fully dehydrated in graded ethanol, critical point dried, and sputter coated with gold. Specimens were examined in a JEOL 840A scanning electron microscope (JEOL, Tokyo, Japan) at 10 kV. In some cases cells were exposed to VEGF (100 ng/ml, R&D Systems, Minneapolis, MN, USA) for 24 h prior to fixation.

Gene expression analysis by focused array

Messenger RNA was extracted from cultured GEnC, and human podocytes used as a control, using an ArrayGrade mRNA purification kit (SuperArray Inc., Bethesda, MD, USA). Gene expression was analysed using a human endothelial cell biology gene array kit (GEArray Q-series, HS-036, SuperArray Inc.). Each array was a 3.8 × 4.8 cm nylon membrane spotted with cDNA fragments of 96 marker genes in quadruplicate. These included four positive controls including glyceraldehyde-3-phosphate dehydrogenase, a negative control, and a range of endothelium-related genes. Briefly, 0.5 μg mRNA was used as template for biotinylated cDNA probe synthesis. RNA was reverse-transcribed using gene-specific primers and cDNA was amplified and labelled with biotin-16-deoxyuridine triphosphate using an AmpoLabelling-LPR Kit (SuperArray Inc.). Biotinylated cDNA probes were denatured and hybridized to the array membranes, which were then washed, blocked, and incubated with alkaline phosphatase-conjugated streptavidin. The hybridized biotinylated probes were detected by a chemiluminescent method using the alkaline phosphatase substrate, CDP-*Star*. The results were analysed using ScanAlyze software (Stanford University, Stanford, CA, USA). Expression levels of individual genes in each mRNA extract were expressed as a ratio of the overall density of the quadruplicate spots representing each gene versus the density of the quadruplicate spots representing glyceraldehyde-3-phosphate dehydrogenase, after background correction. The gene array data on selected endothelial-specific markers were validated by immunofluorescence and Western blotting.

Immunofluorescence

Cells grown to confluence on collagen (Autogen Bioclear, Calne, Wilts., UK)-coated glass coverslips were fixed in 2% formaldehyde and permeabilized in 0.3% Triton X-100. Cells were incubated with blocking solution (5% foetal calf serum and 0.05% Tween 20) and then with antibodies to PECAM1 (R&D Systems), VE-cadherin (Santa Cruz Biochemicals, Santa Cruz, CA, USA), vWF (Dako-Cytomation, Ely, Cams, UK), and SV40LT (Pharmingen, San Diego, CA, USA). Primary antibody binding was detected using fluorescein isothiocyanate-conjugated secondary antibodies (Jackson Immuno Research Laboratories Inc, West Grove, PA, USA). Podocytes were

used as controls. Coverslips were mounted in Vectashield aqueous mountant (Vector Laboratories, Peterborough, UK) and examined using a Leica DMRB fluorescence microscope (Leica, Solms, Germany).

Cell-based fluorescence immunoassay

A fluorescence immunoassay, as described previously,[24] was used to quantify changes in E-selectin expression in response to TNFα. CiGEnC at 33°C were seeded in 96-well plates at 10 000 cells/well. After 5 days cells were transferred to 37°C and after a further 7 days were treated with TNFα (0–10 ng/ml, R&D Systems) for 6 h. The cells were fixed and permeabilized as above, then incubated with an antibody to E-selectin (R&D Systems), which was detected with a fluorescein isothiocyanate-labelled secondary antibody as above. An irrelevant primary antibody was used as a control. Fluorescence emission at 520 nm after excitation at 490 nm was measured on a Packard Instruments FluoroCount fluorospectrophotometer (Perkin-Elmer Life Sciences, Boston, MA, USA).

Western blotting

CiGEnC at 33°C or after 10 days at 37°C were lysed in Laemmli sample buffer and solubilized protein concentrations were determined (bicinchoninic acid assay; Pierce Chemical Co., Rockford, IL, USA). Lysates of primary culture GEnC, podocytes and sieved glomeruli (as described previously[32]) were used as controls. Protein samples were separated by electrophoresis under reducing conditions and were blotted onto nitrocellulose membranes. The membranes were immunolabelled with antibodies to vWF, VE-cadherin, SV40LT (as above), Tie2, VEGFR2 (both Santa Cruz), ICAM2 (R&D Systems), and actin (Sigma). Bands were detected by enhanced chemiluminescence (Amersham Biotech Ltd., Bucks., UK). In other experiments, ciGEnC were lysed at successive time points and SV40LT expression analysed as above.

Measurement of TEER

TEER is a measure of ion flux and is inversely related to the fractional area of pathways open to water and small molecules across a cell monolayer. Tissue culture inserts containing polycarbonate supports (0.4 μm pore size, Nalge Nunc International, Rochester, NY, USA) were seeded with ciGEnC at 100 000 cells/cm^2. Measurement of TEER of GEnC monolayers was performed as described previously using an Endohm 12 electrode chamber and EVOMx voltometer (World Precision Instruments, Sarasota, FL, USA).[24] TEER was measured sequentially in ciGEnC monolayers at 33°C. Some inserts were transferred to 37°C on day 6 after seeding with continued monitoring of TEER.

Effects of cAMP and thrombin on ciGEnC monolayers

The effects of a cAMP analogue and thrombin were examined as increasing intracellular cAMP decreases permeability in primary culture GEnC and other EnC, while thrombin has opposite effects.[24,35] Cell membranes are impermeable to cAMP, so a cell membrane-permeable cAMP analogue, 8-(4-chlorophenylthio)-cAMP (Sigma), was used in combination with a cAMP-specific phosphodiesterase inhibitor, RO-20-1724 (CN Biosciences, Nottingham, UK). Inserts containing ciGEnC monolayers maintained at 37°C for 7 days were used. Culture medium in both wells and inserts was replaced with serum-free medium. Baseline TEER was measured after 1 h and the culture medium was again replaced, this time with serum-free medium containing 20 μM RO-20-1724 and 300 μM 8-(4-chlorophenylthio)-cAMP ('cAMP medium') or 1 U/ml thrombin (Sigma) or with medium alone (control). TEER was remeasured after 1 h.

Statistics

Microsoft Excel software (Microsoft Corporation, Washington, DC, USA) was used for simple statistics. SPSS 11.0 (SPSS, Chicago, IL, USA) was used for other tests, including standard error (s.e.). *P*-values of <0.05 were taken to indicate the statistical significance.

ACKNOWLEDGMENTS

We thank Dr D Griffiths, Dept of Pathology, University of Wales College of Medicine, Heath Park, Cardiff, UK for assistance with electron microscopy and Thea J van der Velden, Radboud University Nijmegen Medical Centre, Nijmegen, and Jess Turnbull, Academic Renal Unit, University of Bristol for technical assistance. Funding was provided by the National Kidney Research Fund (UK) grant R22/1/2003. SCS was funded by Wellcome Trust grant 075731.

REFERENCES

1. Endlich K, Kriz W, Witzgall R. Update in podocyte biology. *Curr Opin Nephrol Hypertens* 2001; **10**: 331–340.
2. Ohlson M, Sorensson J, Haraldsson B. A gel-membrane model of glomerular charge and size selectivity in series. *Am J Physiol Renal Physiol* 2001; **280**: F396–F405.
3. Deen WM, Lazzara MJ, Myers BD. Structural determinants of glomerular permeability. *Am J Physiol Renal Physiol* 2001; **281**: F579–F596.
4. Rostgaard J, Qvortrup K. Electron microscopic demonstrations of filamentous molecular sieve plugs in capillary fenestrae. *Microvasc Res* 1997; **53**: 1–13.
5. Brenchley P. VEGF/VPF: a modulator of microvascular function with potential roles in glomerular pathophysiology. *J Nephrol* 1996; **9**: 10–17.
6. Deen WM. What determines glomerular capillary permeability? *J Clin Invest* 2004; **114**: 1412–1414.
7. van Setten PA, van Hinsbergh VW, van der Velden TJ *et al.* Effects of TNF alpha on verocytotoxin cytotoxicity in purified human glomerular microvascular endothelial cells. *Kidney Int* 1997; **51**: 1245–1256.
8. Maynard SE, Min JY, Merchan J *et al.* Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003; **111**: 649–658.
9. Iruela-Arispe L, Gordon K, Hugo C *et al.* Participation of glomerular endothelial cells in the capillary repair of glomerulonephritis. *Am J Pathol* 1995; **147**: 1715–1727.
10. Masuda Y, Shimizu A, Mori T *et al.* Vascular endothelial growth factor enhances glomerular capillary repair and accelerates resolution of experimentally induced glomerulonephritis. *Am J Pathol* 2001; **159**: 599–608.
11. Shimizu A, Masuda Y, Mori T *et al.* Vascular endothelial growth factor165 resolves glomerular inflammation and accelerates glomerular capillary repair in rat anti-glomerular basement membrane glomerulonephritis. *J Am Soc Nephrol* 2004; **15**: 2655–2665.
12. Deckert T, Feldt-Rasmussen B, Borch-Johnsen K *et al.* Albuminuria reflects widespread vascular damage. The Steno hypothesis. *Diabetologia* 1989; **32**: 219–226.
13. Pedrinelli R, Dell'Omo G, Penno G *et al.* Non-diabetic microalbuminuria, endothelial dysfunction and cardiovascular disease. *Vasc Med* 2001; **6**: 257–264.
14. Hyink DP, Tucker DC, St John PL *et al.* Endogenous origin of glomerular endothelial and mesangial cells in grafts of embryonic kidneys. *Am J Physiol* 1996; **270**: F886–F899.
15. Woolf AS, Loughna S. Origin of glomerular capillaries: is the verdict in? *Exp Nephrol* 1998; **6**: 17–21.
16. Page C, Rose M, Yacoub M *et al.* Antigenic heterogeneity of vascular endothelium. *Am J Pathol* 1992; **141**: 673–683.
17. Kelly JJ, Moore TM, Babal P *et al.* Pulmonary microvascular and macrovascular endothelial cells: differential regulation of Ca^{2+} and permeability. *Am J Physiol* 1998; **274**: L810–L819.
18. Ghitescu L, Robert M. Diversity in unity: the biochemical composition of the endothelial cell surface varies between the vascular beds. *Microsc Res Tech* 2002; **57**: 381–389.
19. Saleem MA, O'Hare MJ, Reiser J *et al.* A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol* 2002; **13**: 630–638.

20. Ballermann BJ, Marsden PA. Endothelium-derived vasoactive mediators and renal glomerular function. *Clin Invest Med* 1991; **14**: 508–517.
21. Wolf G, Ziyadeh FN, Zahner G *et al.* Angiotensin II is mitogenic for cultured rat glomerular endothelial cells. *Hypertension* 1996; **27**: 897–905.
22. Sorensson J, Bjornson A, Ohlson M *et al.* Synthesis of sulfated proteoglycans by bovine glomerular endothelial cells in culture. *Am J Physiol Renal Physiol* 2003; **284**: F373–F380.
23. Rops AL, van der Vlag J, Jacobs CW *et al.* Isolation and characterization of conditionally immortalized mouse glomerular endothelial cell lines. *Kidney Int* 2004; **66**: 2193–2201.
24. Satchell SC, Anderson KL, Mathieson PW. Angiopoietin 1 and vascular endothelial growth factor modulate human glomerular endothelial cell barrier properties. *J Am Soc Nephrol* 2004; **15**: 566–574.
25. Giannini S, Cresci B, Pala L *et al.* Human glomerular endothelial cells IGFBPs are regulated by IGF-I and TGF-beta1. *Mol Cell Endocrinol* 1999; **154**: 123–136.
26. Cresci B, Giannini S, Pala L *et al.* AT1 and AT2 receptors in human glomerular endothelial cells at different passages. *Microvasc Res* 2003; **66**: 22–29.
27. Ott MJ, Olson JL, Ballermann BJ. Chronic *in vitro* flow promotes ultrastructural differentiation of endothelial cells. *Endothelium* 1995; **3**: 21–30.
28. O'Hare MJ, Bond J, Clarke C *et al.* Conditional immortalization of freshly isolated human mammary fibroblasts and endothelial cells. *Proc Natl Acad Sci USA* 2001; **98**: 646–651.
29. Salih V, Knowles JC, O'Hare MJ *et al.* Retroviral transduction of alveolar bone cells with a temperature-sensitive SV40 large T antigen. *Cell Tissue Res* 2001; **304**: 371–376.
30. Jat PS, Sharp PA. Cell lines established by a temperature-sensitive simian virus 40 large-T-antigen gene are growth restricted at the nonpermissive temperature. *Mol Cell Biol* 1989; **9**: 1672–1681.
31. Eremina V, Quaggin SE. The role of VEGF-A in glomerular development and function. *Curr Opin Nephrol Hypertens* 2004; **13**: 9–15.
32. Satchell SC, Harper SJ, Tooke JE *et al.* Human podocytes express angiopoietin 1, a potential regulator of glomerular vascular endothelial growth factor. *J Am Soc Nephrol* 2002; **13**: 544–550.
33. Hillege HL, Fidler V, Diercks GF *et al.* Urinary albumin excretion predicts cardiovascular and noncardiovascular mortality in general population. *Circulation* 2002; **106**: 1777–1782.
34. Saleem MA. Biology of the human podocyte. *Nephron Exp Nephrol* 2003; **95**: e87–e92.
35. He P, Zeng M, Curry FE. Dominant role of cAMP in regulation of microvessel permeability. *Am J Physiol Heart Circ Physiol* 2000; **278**: H1124–H1133.